UNIVERSIDADE FEDERAL DO PARANÁ

FELLIP RODRIGUES MARCONDES

# O PROCESSO DE INVASÃO DE *LIMNOPERNA FORTUNEI* NA BACIA DO RIO IGUAÇU: UM PANORAMA QUASE 10 ANOS APÓS PRIMEIRA AVALIAÇÃO

# THE INVASION PROCESS OF *LIMNOPERNA FORTUNEI* IN THE IGUAÇU RIVER BASIN: AN OVERVIEW ALMOST 10 YEARS OF THE FIRST EVALUTION

CURITIBA

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Dissertação apresentada ao curso de Pós-Graduação em Ecologia e Conservação, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Ecologia.

Orientador: Prof. Dr. Walter Antônio Pereira Boeger.

Coorientador: Dr. Rafael Antunes Baggio

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**THE INVASION PROCESS OF LIMNOPERNA FORTUNEI IN THE IGUAÇU RIVER BASIN: AN OVERVIEW ALMOST 10 YEARS OF THE FIRST EVALUTION**, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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RAFAEL ANTUNES BAGGIO
Presidente da Banca Examinadora

Assinatura Eletrônica
04/08/2025 11:49:26.0
GUSTAVO ALBERTO DARRIGRAN
Avaliador Externo (FACULTAD CIENCIAS NATURALES Y MUSEO)

Assinatura Eletrônica
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ROBERTO FERREIRA ARTONI
Avaliador Externo (UNIVERSIDADE ESTADUAL DE PONTA GROSSA)

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"E se um alguém com o dom de explicar
o universo através de equações escolhesse
ser professor de literatura?
E se um alguém com o dom de assar pães
extraordinários escolhesse ser engenheiro?
Quando o dom divino de uma pessoa
diverge do que ela quer fazer, qual será o
caminho que trará a verdadeira felicidade a ela?"
(Hiroshi Sakurazuka & Takeshi Obata. *All you need is Kill*. Editora JBC. 2015)

"Quem tenta imaginar a perfeição, simplesmente revela seu próprio vazio."
(George Orwell, Carta ao *Tribune*, 1943)

## RESUMO

O mexilhão-dourado, *Limnoperna fortunei* (Dunker, 1857), é um molusco bivalve invasor de origem asiática que se disseminou rapidamente nas últimas décadas, causando impactos ecológicos e econômicos significativos. Este estudo investigou a diversidade genética e a estrutura populacional de *L. fortunei* na bacia do Baixo Rio Iguaçu e no reservatório de Itaipu (Bacia do Alto Rio Paraná), utilizando SNPs (*Single Nucleotide Polymorphisms*) por meio de Genotipagem por Sequenciamento (GBS), com os seguintes objetivos: (1) caracterizar o cenário genético atual das populações; e (2) comparar os resultados com dados prévios literatura, para avaliar mudanças temporais. Foram coletados indivíduos em quatro locais: UHE Salto Caxias, montante e jusante da UHE Baixo Iguaçu e reservatório da UHE Itaipu. As análises revelaram três padrões principais: (i) alta conectividade genética entre as populações do Baixo Iguaçu, com ausência de estruturação significativa, indicando intenso fluxo gênico mediado por dispersão hidrológica natural e transporte antrópico; (ii) nítida diferenciação genética entre as populações do Iguaçu e Itaipu, evidenciando o efeito de barreira das Cataratas do Iguaçu na limitação do fluxo gênico entre estas bacias; e (iii) dinâmicas populacionais distintas entre os sistemas, com fluxo gênico bidirecional no Iguaçu (especialmente na região da UHE Baixo Iguaçu) e menor conectividade com Itaipu. A comparação temporal com Borges et al. (2017) indica padrões persistentes de conectividade ao longo do Iguaçu, mas com diferenciação sutil entre subpopulações, sugerindo dispersão contínua sem novas introduções externas, além de demonstrar que as Cataratas do Iguaçu permanecem como barreira efetiva entre bacias. Esses achados reforçam a importância de considerar tanto fatores naturais quanto antropogênicos na dinâmica populacional desta espécie invasora em ambientes fragmentados, auxiliando no processo de criação de estratégias de manejo, como controle de vetores antrópicos entre reservatórios e monitoramento genético para detecção de mudanças nos padrões de dispersão.



**Palavras-chave**: Diversidade genética; GBS; Invasões Biológicas; Mexilhão-dourado; Mytilidae.

## ABSTRACT

The golden mussel, *Limnoperna fortunei* (Dunker, 1857), is an invasive Asian bivalve mollusk that has rapidly spread in recent decades, causing significant ecological and economic impacts. This study investigated the genetic diversity and population structure of *L. fortunei* in the Lower Iguaçu River basin and the Itaipu Reservoir (Upper Paraná River basin), using Single Nucleotide Polymorphisms (SNPs) through Genotyping-by-Sequencing (GBS), with the following objectives: (1) to characterize the current genetic scenario of the populations; and (2) to compare the results with previous literature data to assess temporal changes. Individuals were collected at four sites: Salto Caxias Hydroelectric Power Plant (HPP), upstream and downstream of Baixo Iguaçu HPP, and the Itaipu HPP reservoir. The analyses revealed three main patterns: (i) high genetic connectivity among Baixo Iguaçu populations, with no significant structure, indicating intense gene flow mediated by natural hydrological dispersal and anthropogenic transport; (ii) clear genetic differentiation between Iguaçu and Itaipu populations, demonstrating the barrier effect of Iguaçu Falls in limiting gene flow between these basins; and (iii) distinct population dynamics between systems, with bidirectional gene flow in the Iguaçu (especially in the Baixo Iguaçu HPP region) and lower connectivity with Itaipu. The temporal comparison with Borges et al. (2017) shows persistent connectivity patterns along the Iguaçu, but with subtle differentiation among subpopulations, suggesting continuous dispersal without new external introductions, while confirming that Iguaçu Falls remains an effective barrier between basins. These findings reinforce the importance of considering both natural and anthropogenic factors in the population dynamics of this invasive species in fragmented environments, supporting the development of management strategies such as controlling anthropogenic vectors between reservoirs and genetic monitoring to detect changes in dispersal patterns.



**Keywords**: Genetic diversity; GBS; Biological Invasions; Golden Mussel; Mytilidae.

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**SUMÁRIO**

# 1 INTRODUÇÃO GERAL

O mexilhão dourado (*Limnoperna fortunei*, Dunker 1857) é um molusco bivalve pertencente à família Mytilidae, originário do Sudeste Asiático, que foi introduzido na América do Sul, na década de 1990, muito provavelmente por meio da água de lastro de navios mercantes (Darrigran & Pastorino, 1995, Darrigran, 1997). Os primeiros registros desta espécie no continente ocorreram no estuário do Rio de La Plata, na Argentina, em 1991 (Pastorino et al., 1993; Darrigran & Pastorino, 1995). Desde então, a espécie colonizou diversos ambientes naturais e artificiais, expandindo sua distribuição por milhares de quilômetros pela América do Sul, em países como Uruguai (1994), Paraguai (1997), Brasil (1998) e Bolívia (Pastorino et al., 1993; Darrigran, 2002; Darrigran & Pastorino, 2004; Boltovskoy et al., 2006, Brugnolli et al., 2011; Campos et al., 2014). No Brasil, o primeiro registro ocorreu em 1998, na bacia do alto rio Paraguai, próximo à cidade de Cáceres, Mato Grosso, sendo também registrado no mesmo ano no delta do Rio Jacuí, no Rio Grande do Sul (Mansur et al., 2003; Darrigran & Damborenea, 2009, Darrigran et al., 2020).

A dispersão de *L. fortunei* pela América do Sul tem sido impulsionada por diferentes mecanismos, sendo que muitos deles estão relacionados com atividades antrópicas (Darrigran, 2002; Boltovyskoy et al., 2006; Belz et al., 2012). A deriva larval a jusante permite a colonização rápida de novos habitats, enquanto a dispersão a montante ocorre principalmente pela bioincrustação em embarcações e equipamentos náuticos, assim como o transporte de areia do fundo dos rios para outras localidades (Boltovskoy et al., 2006; Karatayev et al., 2007; Belz et al., 2012). Segundo Zhan et al. (2012), pequenas embarcações têm sido um dos principais vetores da propagação desse invasor, possibilitando a ocorrência de dispersão por "saltos" entre diferentes bacias hidrográficas. Como resultado, *L. fortunei* foi registrado em diversos sistemas aquáticos do Brasil, desde o delta do rio Jacuí, no Rio Grande do Sul até a bacia do Rio São Francisco (Mansur et al., 2003; Barbosa et al., 2016).

## 1.1 BIOLOGIA E ECOLOGIA DE *L. FORTUNEI*

O sucesso da invasão do mexilhão-dourado na América do Sul ocorreu devido a uma série de capacidades biológicas e ecológicas. Suas conchas oferecem proteção contra variações ambientais, como flutuações de temperatura, salinidade, pH e predação (Darrigran, 2002; Uliano-Silva et al., 2016). Além disso, a espécie exibe notável plasticidade fenotípica, permitindo ajustes morfológicos e fisiológicos em resposta às mudanças no ambiente (Darrigran & Damborenea, 2009; Uliano-Silva et al., 2016).

Reprodutivamente, *L. fortunei* é gonocórico (com sexos separados), apresenta fecundação externa e desenvolvimento larval indireto, características que favorecem sua rápida proliferação (Cataldo, 2005). Suas larvas, dispersas principalmente pela água, contribuem para a colonização de novos habitats, enquanto a ausência de predadores naturais nas regiões invadidas potencializa seu sucesso (Darrigran & Damborenea, 2009, Giglio et al., 2016). Quando adultos, fixam-se em diferentes tipos de substratos graças a filamentos proteicos chamados de bisso, que garantem sua adesão, formando densos aglomerados, amplificando seus impactos (Darrigran & Damborenea, 2009).

A introdução de espécies exóticas é um dos principais fatores de perda de biodiversidade, pois altera a composição e funcionalidade dos ecossistemas (IUCN, 2000; Xu et al., 2006). Muitas atividades humanas, como transporte marítimo, aquicultura, recreação e construção de canais, favorecem a dispersão de organismos além de suas barreiras naturais (Ruiz et al., 1997). No caso de *L. fortunei*, sua presença em novos ambientes tem resultado em impactos ecológicos severos, como a alteração na dinâmica de ecossistemas aquáticos, promovendo um crescimento acelerado de fitoplâncton e cianobactérias, reduzindo turbidez e modificando ciclos de nutrientes (Boltovskoy et al., 2006). Além disso, compete com espécies nativas por recursos e espaço, fixando-se em moluscos e crustáceos, o que pode levar a desequilíbrios na cadeia alimentar (Cataldo et al., 2005; Frau et al., 2013; Darrigran et al., 2020).

## 1.2 METODOLOGIAS PARA MONITORAMENTO, CONTROLE E MITIGAÇÃO DO IMPACTO DE *L. FORTUNEI*

O monitoramento do mexilhão-dourado com base em características morfológicas é limitado, especialmente em estágios larvais, pois muitas características distintivas só se tornam visíveis em fases avançadas de desenvolvimento (Garland & Zimmer, 2002). Além disso, fatores ambientais podem influenciar traços morfológicos, como variações na estrutura da concha e brânquias, dificultando a distinção entre espécies (Pie et al., 2006; Scheffers et al., 2016; Stange et al., 2020). Apesar dessas limitações, a morfometria ainda é útil para estudos de plasticidade fenotípica, já que *L. fortunei* exibe adaptações morfológicas em resposta a gradientes ambientais, como mudanças na proporção largura-comprimento da concha (Peyer et al., 2010). No entanto, métodos puramente ópticos, como microscopia, são pouco eficientes para detecção de larvas em amostras de plâncton, exigindo técnicas complementares para confirmação (Boeger et al., 2007).

A biologia molecular revolucionou a detecção de *L. fortunei*, especialmente em amostras de plâncton. Para isto, foram desenvolvidos marcadores moleculares específicos, utilizando um fragmento de DNA mitocondrial, com discriminação específica (Pie et al., 2006; Boeger et al., 2007). Posteriormente, foram desenvolvidas técnicas de detecção de DNA ambiental (eDNA), as quais detectam o material genético no ambiente, sem que haja a necessidade de coleta ou captura de organismos, o que tornou esta técnica uma ferramenta de baixo custo e fundamental para detecção inicial de espécies invasoras (Rees et al., 2014, Pie et al., 2017; Andrade et al., 2021). A detecção precoce é vital, pois intervenções no início do processo invasivo são mais eficazes para evitar estabelecimento populacional (Zanden & Olden, 2008).

Para estudos populacionais, uma das técnicas comumente utilizadas para avaliar a dinâmica populacional e o rastreio das rotas de invasão dos mexilhão-dourado são os marcadores de microssatélites, que são caracterizados pela repetição de pequenas unidades nucleotídicas e apresentam altos níveis de polimorfismos entre indivíduos, (Zane et al., 2002; Ellegren, 2004; Zhan et al., 2012; Abdul-Munner, 2014).

Mais recentemente, o sequenciamento de Nova Geração (NGS, *Next-Generation Sequencing*) abriu uma nova gama de oportunidades para o monitoramento do mexilhão-dourado, permitindo uma compreensão mais profunda dos organismos avaliados e das suas interações genéticas (Rius et al., 2015). A Genotipagem por Sequenciamento (GBS - *Genotyping By Sequencing*) é uma das técnicas de nova geração que identifica Polimorfismo de Nucleotídeo Único (SNPs - *Single Nucleotide Polymorphism*) em larga escala, oferecendo alta resolução para estudos genômicos (Elshire et al., 2011; Torkamaneh et al., 2016). Com esta ferramenta, é possível monitorar dispersões, identificar rotas de invasão e populações fundadoras, permitindo a expansão dos dados em relação a outros métodos, como os microssatélites (Ferreira et al., 2020). Com relação à custo-efetividade, comparado a outras ferramentas da nova geração, o GBS é mais acessível para análises em larga escala, fornecendo dados para políticas de manejo (Torkamaneh et al., 2016).

## 1.3 BACIAS DO RIO IGUAÇU E DO RIO PARANÁ

As bacias hidrográficas do Rio Iguaçu e do Rio Paraná representam sistemas fluviais de extrema importância para o desenvolvimento socioeconômico e a conservação ambiental na América do Sul. O Rio Iguaçu, com seus aproximadamente 1.320 km de extensão, nasce na Serra do Mar no estado do Paraná e deságua no Rio Paraná, na região de fronteira entre Brasil, Argentina e Paraguai (ANA, 2020). Sua bacia, que cobre cerca de 70 mil km² quase

inteiramente dentro do território paranaense, é mundialmente conhecida pelas Cataratas do Iguaçu, um dos maiores conjuntos de quedas d'água do planeta e importante polo turístico internacional (ICMBio, 2019). O Rio Paraná se destaca como um dos cursos d'água mais extensos do continente, com 4.880 km desde sua formação pela confluência dos rios Paranaíba e Grande, em Minas Gerais até sua foz no Rio de La Plata (ANA, 2020). Percorrendo vários estados brasileiros e servindo de fronteira natural com Paraguai e Argentina, sua bacia hidrográfica de 2,8 milhões de km² desempenha papel estratégico no abastecimento de água, navegação, pesca e, especialmente, na geração de energia elétrica para estes países, a partir do complexo de Itaipu, a maior usina hidrelétrica do mundo em capacidade instalada (ANEEL, 2021). Além disso, o turismo nas Cataratas do Iguaçu movimenta anualmente mais de 1 bilhão de reais, enquanto as atividades pesqueiras comerciais e esportivas sustentam comunidades ribeirinhas e movimentam a economia regional (ICMBio, 2019).

No rio Iguaçu, um afluente do rio Paraná, o primeiro registro da espécie ocorreu em 2001, em dois pontos próximos à cidade de Curitiba, Paraná (Takeda et al., 2003). No mesmo ano, foi detectado na Usina Hidrelétrica (UHE) de Itaipu, localizada no rio Paraná (Borges et al., 2017). Em 2005, larvas da espécie foram identificadas no trecho final do Rio Iguaçu, a jusante das Cataratas do Iguaçu, e no Rio Paraná, em baixas densidades (Pestana et al., 2008). Nos anos subsequentes, o mexilhão-dourado foi registrado em diversos trechos do rio Iguaçu, demonstrando um padrão de dispersão a montante por meio de saltos sucessivos em direção às regiões superiores da bacia hidrográfica (Pestana et al., 2010; Belz et al., 2012; Zhan et al., 2012, Borges et al., 2017). Entretanto, sua presença permaneceu ausente nos trechos urbanos e na nascente do rio, possivelmente em decorrência de condições ambientais inadequadas ao estabelecimento populacional, como alterações físico-químicas associadas à intensa atividade antrópica (Pestana et al., 2010). A propagação rápida e a capacidade de incrustação em superfícies duras naturais e artificiais têm favorecido o sucesso da invasão da espécie, causando impactos ambientais e econômicos significativos, especialmente em usinas hidrelétricas (Darrigran, 2002; Darrigran & Damborenea, 2005; Barbosa et al., 2016).

A invasão do mexilhão-dourado nestas bacias gera uma série de impactos econômicos e ambientais. A nível econômico, devido a sua capacidade de incrustação em estruturas submersas, causa entupimentos em tubulações e sistemas de refrigeração de usinas hidrelétricas, resultando em prejuízos da ordem de US$ 1 milhão em um dia de parada das

turbinas para limpeza em Itaipu e cerca de 40 mil reais em usinas hidrelétricas menores, de 120MW, com três unidades geradoras, sem contabilizar os custos de manutenção e remoção das incrustações (IBAMA, 2020). Estima-se que os custos totais com controle, prevenção e reparos no Brasil fiquem em torno de US$ 6,9 a US$ 9,97 milhões anualmente (Rebelo et al., 2018, Adelino, 2021). A nível ambiental, os danos são igualmente preocupantes, devido à alteração que a espécie causa na qualidade da água, reduzindo a turbidez e aumentando as concentrações de amônia e fosfatos, além da competição de recursos com espécies nativas (Boltovskoy et al., 2006). Outro ponto relevante é que a dispersão do mexilhão que é intensificada por atividades humanas como navegação, piscicultura e transporte de embarcações, exigindo urgentes estratégias de monitoramento e mitigação (Belz et al., 2012).

## 1.4 PERSPECTIVAS E MITIGAÇÃO

A mitigação de *L. fortunei* representa um desafio complexo, exigindo estratégias integradas que combinem monitoramento precoce, controle químico e biológico, e gestão de vetores de dispersão. Atualmente, não existe um método padrão universalmente eficaz para seu controle em diferentes ambientes (Rajagopal et al., 1991). O uso de cloro e outros biocidas, por exemplo, mostra limitações devido à capacidade dos mexilhões de fechar suas conchas em resposta a toxinas (Calazans et al., 2013). Soluções inovadoras, tais como a microencapsulação de biocidas, têm sido exploradas para aumentar a eficácia desses compostos, liberando-os de forma controlada no ambiente (Calazans et al., 2013).

Alternativas promissoras incluem controle genético, inspirado em modelos como o mosquito *Aedes aegypti* geneticamente modificado, que reduz populações-alvo por meio de letalidade condicional (Phuc et al., 2007). Embora ainda em fase experimental para *L. fortunei*, essa abordagem enfrenta desafios, como a produção de embriões em escala e riscos ecológicos associados à liberação de organismos modificados (Rebelo et al., 2018).

A gestão de vetores é igualmente crítica. Atividades humanas — como transporte de areia, navegação recreativa e aquicultura — são os principais responsáveis pela dispersão do mexilhão (Ruiz et al., 1997; Belz et al., 2012). Medidas como a quarentena de areia (30 dias antes do uso) e a limpeza de embarcações podem reduzir significativamente o risco de introdução (Belz et al., 2012). Além disso, peixes que ingerem larvas vivas podem atuar como vetores naturais, facilitando a dispersão rio acima (Darrigran, 2002; Belz et al., 2012).

A prevenção permanece a estratégia mais custo-efetiva (Darrigran & Damborenea, 2009; Boltovskoy et al., 2015). Protocolos de avaliação de risco ambiental e regulamentações rigorosas para água de lastro e aquicultura são essenciais (Gherardi, 2007). No entanto, ações

muitas vezes são reativas, implementadas apenas após o estabelecimento da espécie (Ricciardi & Cohen, 2007). Portanto, investir em redes de monitoramento contínuo e cooperação internacional é fundamental para mitigar impactos econômicos e ecológicos, assim como a educação da sociedade, para que compreendam a problemática das bioinvasões (Darrigran et al., 2012).

# 2 CAPÍTULO I – THE INVASION PROCESS OF *LIMNOPERNA FORTUNEI* IN THE IGUAÇU RIVER BASIN: AN OVERVIEW AFTER ALMOST 10 YEARS

Fellip Rodrigues Marcondes[1,2], Rafael A. Baggio[3], Roberto Ferreira Artoni[4], Mateus Henrique Santos[4], Mara Cristina de Almeida Matiello[4], Augusto Ferreira Jr[4], Bruno Henrique Mattiello[5], Giovani Natal dos Santos[5], Leonardo Pussieldi Bastos[2], Luciana Rodrigues de Souza-Bastos[2], Walter A. P. Boeger[1]

[1]Programa de Pós-Graduação em Ecologia e Conservação, Universidade Federal do Paraná, Curitiba, PR, Brasil.

[2]Institutos Lactec, Laboratório de Ecotoxicologia e Avaliação Ambiental, Curitiba, PR, Brasil

[3]Baggio Tecnologia e Meio Ambiente

[4] Laboratório de Genética Evolutiva (LabGEv), Universidade Estadual de Ponta Grossa, Ponta Grossa, PR. Brasil.

[5] Departamento Corporativo de P&D, Neoenergia, S.A

## 2.1 ABSTRACT

*Limnoperna fortunei* (Dunker 1857), an invasive bivalve originating from Asia, has become an ecologically and economically significant bioinvader across South American waterways. Our research examines the population genetics of this golden mussel in two interconnected systems - the Lower Iguaçu River basin and Itaipu Reservoir (Upper Paraná basin) - employing Genotyping-by-Sequencing (GBS) of Single Nucleotide Polymorphisms (SNPs). The study had dual objectives: (1) mapping contemporary genetic patterns across populations, and (2) evaluating decadal changes through comparison with baseline data from Borges et al. (2017). Specimens were sampled from four strategic locations spanning the Salto Caxias HPP, Baixo Iguaçu HPP (upstream and downstream sections), and Itaipu Reservoir. Three fundamental genetic patterns emerged from our analyses: First, Baixo Iguaçu populations showed remarkable genetic homogeneity with minimal substructure, reflecting active gene exchange facilitated by both river currents and human-mediated transport. Second, pronounced genetic divergence between Iguaçu and Itaipu populations confirmed the lasting barrier effect of Iguaçu Falls. Third, we observed asymmetric connectivity patterns, with bidirectional genetic exchange within the Iguaçu system (particularly around Baixo Iguaçu HPP) contrasting with limited Itaipu interactions. Temporal analysis revealed consistent connectivity regimes along the Iguaçu since 2017, though with emerging microdifferentiation among subpopulations - suggesting sustained natural and anthropogenic dispersal without

additional colonization events. The persistent genetic segregation across the falls highlights how major geographic features constrain this invader's spread. These insights into the mussel's metapopulation dynamics in fragmented habitats inform ongoing management efforts, particularly regarding inter-reservoir biosecurity and genetic surveillance protocols

**Keywords**: Genetic diversity; GBS; Biological Invasions; Golden Mussel; Mytilidae.

## 2.1 INTRODUCTION

The invasion process can cause significant changes to the local ecosystem, negatively impacting biodiversity, ecosystem services, and the economy at different scales (Espínola & Julio, 2007; Walther et al., 2009; Anderson et al., 2015; Duchini et al., 2015). Among the observed effects are the reduction of native species diversity, competition for resources such as space and food, the introduction of diseases and vectors, and impacts on human activities (Espínola & Julio, 2007; Walther et al., 2009). Understanding the dispersal dynamics, reproductive cycles, development, and genetic patterns of these species is essential to comprehend the eco-evolutionary processes that influence their colonization and distribution across different geographic scales (Hampton et al., 2004; Wilson et al., 2009). By addressing these elements, it becomes possible to investigate evolutionary factors, such as natural selection and gene flow, that are related to colonization success, establishment in new areas, and population changes over medium and long terms. These are critical components for developing control strategies and local biodiversity conservation, with a focus on minimizing the negative impacts caused by the golden mussel (Hampton et al., 2004; Wilson et al., 2009; Zhan et al., 2012).

*Limnoperna fortunei* (Dunker, 1857), known as the golden mussel, is an Asian-origin bivalve mollusk belonging to the family Mytilidae, which has caused significant impacts in freshwater environments in South America and East Asia (Pastorino et al., 1993; Kimura, 1994; Ricciardi, 1998; Oliveira et al., 2010; Darrigran & Damborenea, 2009; Zhan et al., 2012; Uliano-Silva et al., 2013). The golden mussel has effectively established itself in diverse environments where it is introduced, resulting in multiple ecological impacts, such as reduced biodiversity in invaded natural areas, competition for resources, and altered water quality (Darrigran et al., 1998; Darrigran & Damborenea, 2009; Avelino et al., 2019; IBAMA, 2020). Beyond environmental impacts, *L. fortunei* is responsible for economic losses in

sectors including aquaculture, navigation, water supply, tourism, and particularly the energy sector (IBAMA, 2020; Taveira et al., 2024). In the energy sector, damages include pipeline obstruction, filter clogging, reduced flow rates, and decreased cooling system efficiency, generating estimated annual costs in Brazil of US$9.97 million for prevention and control, plus US$6.9 to 8 million for monitoring and maintenance (Rebelo, 2018; Adelino, 2021).

The golden mussel exhibits gregarious and biofouling behavior, attaching to both natural and artificial substrates during its adult phase through the byssus, an anchoring structure composed of protein filaments secreted by the muscular foot and terminated with an adhesive plaque that facilitates attachment (Darrigran & Damborenea, 2009; Li et al., 2018). Its life cycle consists of three well-defined periods: larval, juvenile, and adult, with the larval and juvenile stages being planktonic (Darrigran et al., 1998; Cataldo, 2015). At the end of the juvenile phase, upon reaching sexual maturity, these organisms attach to a substrate (Darrigran & Damborenea, 2009; Cataldo, 2015).

The passive dispersal of the golden mussel occurs predominantly through jump dispersal, a pattern characteristic of invasive species with planktonic stages (Zhan et al., 2012). This dynamic is mediated by anthropogenic vectors, including navigation of small vessels that transport larvae via residual water or hull fouling, and aquaculture practices such as equipment translocation or ponds with contaminated water (Belz et al., 2012; Zhan et al., 2012; Cataldo, 2015). Once established, populations may expand naturally downstream through passive larval dispersal via hydrological flow (Darrigran & Damborenea, 2009). Thus, the combination of larval mobility and human activity explains the species' rapid expansion into new habitats (Darrigran & Damborenea, 2009).

Following its introduction to South America in 1991 in the Río de la Plata estuary through ballast water from merchant ships, the golden mussel spread rapidly, being recorded in Brazil in 1998 with accelerated dispersal via multiple vectors, including hull fouling of vessels and aquaculture-related activities (Darrigran & Pastorino, 1995; Boltovskoy et al., 2006; Belz et al., 2012; Borges et al., 2017). Its detection in the Itaipu Hydroelectric Power Plant (HPP Itaipu) reservoir occurred around 2001. In subsequent years, its dispersal into the Iguaçu River was exclusively mediated by anthropogenic vectors, as the Iguaçu Falls represent a geographical barrier to natural dispersal (Belz et al., 2012; Borges et al., 2017). Its introduction to the river occurred through the transport of sand from Itaipu for artificial beach construction at various points along the Iguaçu River, along with dispersal via fouled vessels,

translocation of fish containing live mussels in their digestive tracts, or larvae in fish transport water (Takeda et al., 2003; Boltovskoy et al., 2006; Belz et al., 2012; Borges et al., 2017). The Iguaçu River, located in southern Brazil, is notable for its rich biodiversity, including endemic ichthyofauna (Baumgartner et al., 2012). However, the construction of numerous hydroelectric plants along the river's course has transformed the local ecosystem, creating reservoirs that altered hydrological dynamics and aquatic habitats, impacting native fauna and favoring the proliferation of invasive species like the golden mussel. These populations, due to multiple and continuous introductions, exhibit high genetic variability, requiring constant monitoring to assess their ecological and socioeconomic impacts (Belz et al., 2012; Borges et al., 2017).

Borges et al. (2017) demonstrated that the genetic diversity of *L. fortunei* in the Iguaçu River is consistent with unique or limited introduction events via anthropogenic vectors, leading to a weakly structured population. These findings reinforce the importance of continuous monitoring of genetic variability to assess population viability over time, particularly in environments fragmented by natural barriers (such as the Iguaçu Falls). The comparison between current data and those from Borges et al. (2017) will enable identification of potential changes in genetic diversity over the past decade, elucidating whether the population maintains sufficient gene flow for its persistence or is subject to bottleneck effects or genetic drift.

Within this context, genetic tools emerge as an important approach for understanding species invasion processes and their eco-evolutionary aspects (Boltovskoy et al., 2006; Rius et al., 2008). These tools enable assessment of invasion processes through analysis of genetic variability in individuals and populations, which is modulated by evolutionary processes and mechanisms including demographic characteristics and changes, selection, mutation, and genetic recombination (Scheffers et al., 2016; Stange et al., 2020). Furthermore, ecological changes - typically associated with spatial dispersal processes - occur through a process of ecological fitting (Agosta et al., 2008; Hoberg et al., 2013; Agosta & Brooks, 2020). Ecological fitting is the process by which organisms colonize and persist in new environments, utilize new resources, and/or form new associations with other species as a result of the trait combinations they possess when encountering novel conditions (Janzen, 1985; Agosta et al., 2008). Therefore, introduced populations depend on both the genetic and epigenetic characteristics inherited from the source population, as well as their phenotypic

plasticity. Collectively, these factors enhance their establishment potential (Lee, 2002; Laikre, 2010; Handley et al., 2011).

Various molecular markers have been widely employed to infer the nature of genetic diversity and the evolutionary and demographic processes of invasive populations (Rius & Darling, 2014; Turchetto-Zolet et al., 2017). These data are fundamental for understanding population dynamics and the evolutionary mechanisms that inform both invasive species control and conservation strategy development (Roman & Darling, 2007; Rius et al., 2015). Single Nucleotide Polymorphism (SNP) is a widely used genetic marker for studies of genetic diversity, population structure, and gene flow (Elshire et al., 2011). For large-scale SNP genotyping, the Genotyping-by-Sequencing (GBS) technique stands out as a robust tool, enabling precise and sensitive assessment of thousands of genomic sites at reduced costs (Elshire et al., 2011; Torkamaneh et al., 2016). Due to these characteristics, GBS has proven highly effective for studying populations of invasive species, such as the golden mussel.

Given this scenario, the present study aims to investigate the current genetic landscape of *Limnoperna fortunei* populations in the Lower Iguaçu River basin and the Itaipu Reservoir (Upper Paraná River basin) using thousands of SNPs to: (i) assess their genetic diversity and gene flow patterns; and (ii) compare these results with those of Borges et al. (2017), identifying potential changes in population structure and genetic variability over this decade - resulting from both the novel methodology and ecological/anthropogenic processes.

## 2.3 MATERIAL AND METHODS

### 2.3.1 Sampling

Specimens of *L. fortunei* were collected from the Itaipu Reservoir, located in the Upper Paraná River basin (25.4470° S, 54.5495° W), and from three locations in the Lower Iguaçu River basin: the Salto Caxias Hydroelectric Power Plant (HPP Salto Caxias) reservoir (-25.5408° S, -53.4112° W), upstream of the Baixo Iguaçu HPP reservoir (-25.5192° S, -53.6374° W), and downstream of the Baixo Iguaçu HPP reservoir (-25.5072° S, -53.6794° W) (Figure 1). A total of 50 individuals per site (19 for the downstream Baixo Iguaçu HPP population) were manually sampled between May 2023 and August 2023.

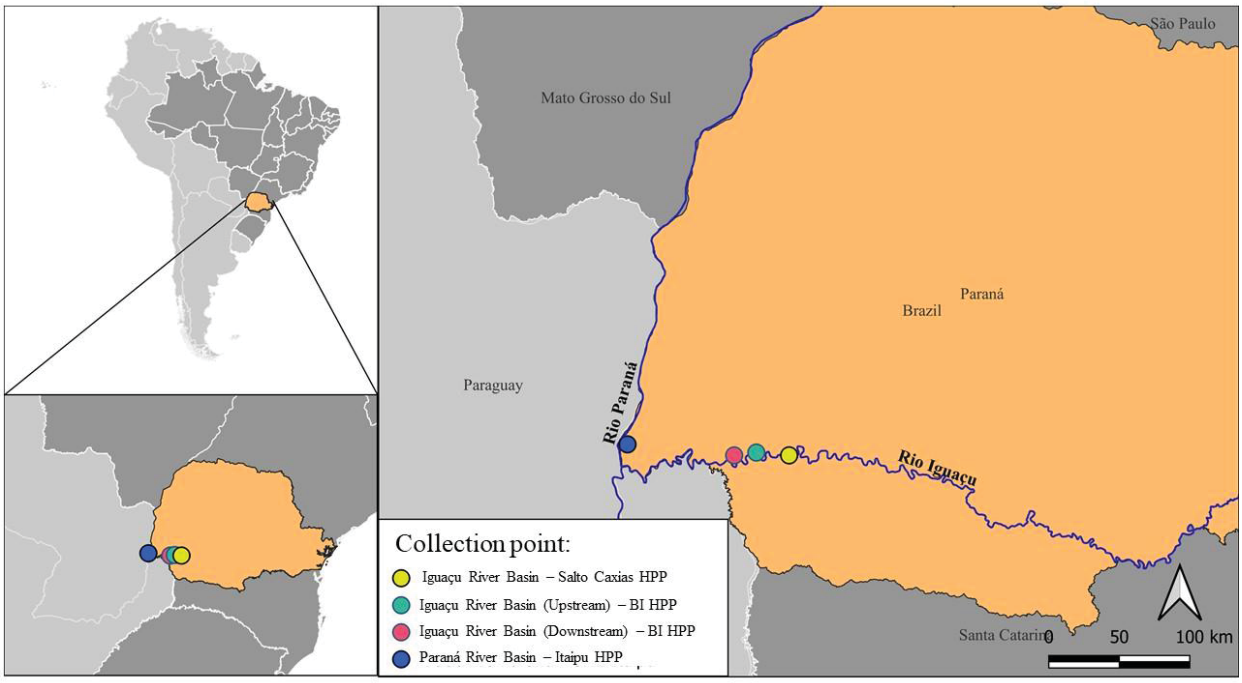


**Figure 1.** Collection points for *Limnoperna fortunei* in the Lower Iguaçu River basin and Upper Paraná River basin. Salto Caxias HPP (yellow); Baixo Iguaçu Upstream HPP (green); Baixo Iguaçu Downstream HPP (red); Itaipu HPP (blue). Source: The Author.

Following collection, the soft tissues of specimens were removed from their shells and individually stored in 2 ml cryotubes containing 96% ethanol. The samples were subsequently transported to the Environmental Toxicology and Assessment Laboratory at Lactec Institute in Curitiba, Paraná State, where they were stored at -80°C until genetic material extraction.

### 2.3.2 Sample preparation and processing

The genomic DNA (gDNA) from each individual was extracted using the Wizard® Genomic DNA Purification Kit (Promega), with protocol modifications including: (1) addition of 120 µL of 0.5 M EDTA, (2) use of 3 µL RNAse, and (3) 20 µL Proteinase K. Following extraction, gDNA concentration and integrity were assessed using: (i) a Qubit 4® Fluorometer (Invitrogen) and (ii) 0.8% agarose gel electrophoresis stained with 20× GelRed® fluorophore (Biotium), with visualization under UV-II illumination at 302 nm using a UV transilluminator (Kasvi®).

From the 50 individuals that had their DNA extracted from each population, those with the best DNA concentration and integrity results were selected for SNP genotyping. The chosen samples included 25 individuals from the Salto Caxias HPP location, 24 from the upstream Baixo Iguaçu HPP location, 19 from the downstream Baixo Iguaçu HPP location, and 24 from the Itaipu HPP. All 92 samples were normalized to 20 ng/µL in 100 µL aliquots.

### 2.3.3 SNPs Genotyping

SNP genotyping was performed using the Genotyping-by-Sequencing (GBS) technique, which involved the preparation and sequencing of DNA libraries. The gDNA was digested with the PstI enzyme, followed by ligation of specific barcodes (unique, predefined nucleotide sequences used as molecular markers for identifying and differentiating biological species or molecules) to the generated fragments. The DNA libraries were then prepared, amplified by Polymerase Chain Reaction (PCR), purified using magnetic beads, and quality was assessed using the Bioanalyzer 2100 (Agilent) and qPCR (real-time PCR). Sequencing was performed on a Next Generation Sequencing (NGS) platform (Illumina HiSeq 2500), generating approximately 200 million single-end 125 bp reads.

### 2.3.4 Assembly and filtering of SNPs

The sequencing data were processed for SNP panel assembly and filtering. Initially, sequencing quality and contaminant presence were assessed using FastQC (Andrews, 2010). Sequences were demultiplexed according to their barcodes using the *process_radtags* module in Stacks 2.62 (Catchen et al., 2011, 2013), removing degenerate sequences and those lacking the restriction enzyme. Illumina adapters and low-quality ends ($q < 10$) were trimmed using cutadapt 1.8.3 (Martin, 2011), and low-quality sequences were filtered out in *process_radtags*.

The SNP panels were assembled using the *reference-based* pipeline in Stacks 2.62 (Rochette & Catchen, 2017). Sequences were aligned to the *L. fortunei* reference genome

(GenBank accession GCA_944474755.1) using the BWA-MEM algorithm in BWA (Li & Durbin, 2010). The *gstacks* and *population*s modules in Stacks 2.62 identified and filtered SNPs, retaining only those present in 50% of individuals, with maximum heterozygosity of 0.75 and minimum allele frequency of 2%. When multiple SNPs were present in a single fragment, one SNP was randomly selected for analysis.

Due to the trade-off between the number of individuals and filtered SNPs, two databases were created for population analyses. In genetic database A (BGA), we prioritized more SNPs and fewer individuals. In genetic database B (BGB), we prioritized more individuals and fewer SNPs. Using R 4.2.3 (R Core Team, 2023), SNPs were removed through interactive filtering with excessive missing data proportions (above 0.5 for BGA and 0.7 for BGB), along with individuals having more than 0.3 (BGA) and 0.4 (BGB) missing data. Subsequently, alleles with read depth below five copies and frequency under 2% were converted to missing data, while the top 2.5% of SNPs with highest read depth were discarded due to overrepresentation. Following this, additional filtering removed SNPs and individuals with excessive missing data using the same established criteria. To prevent bias in population analyses from potentially physically linked SNPs on the same chromosome, those separated by less than 1,000 base pairs were removed using the SNPfiltR (DeRaad, 2022) and vcfR (Knaus et al., 2017) packages.

The interactive filtering followed the approaches proposed by O'Leary et al. (2018), implemented using the adegenet (Jombart, 2008; Jombart & Ahmed, 2011) and poppr (Kamvar et al., 2014) packages in R 4.2.3. SNPs deviating from Hardy-Weinberg equilibrium and genetic differentiation outliers were removed using the *pegas* (Paradis, 2010) and OutFLANK (Whitlock & Lotterhos, 2014) packages.

### 2.3.5 Analysis of diversity and population structure

The generated SNP panels were used to analyze the genetic diversity, population structure, and gene flow of *L. fortunei*. In R 4.2.3, using the *adegenet* package, we calculated expected (He) and observed (Ho) heterozygosity and missing data percentages, while private allele numbers were estimated with the *poppr* package (Kamvar et al., 2014). Gene diversity and inbreeding coefficients (FIS) were determined in Arlequin 3.5 (Excoffier & Lischer, 2010), and effective population size (Ne) was estimated using the Linkage Disequilibrium method in NeEstimator V2.01 (Do et al., 2014).

Fst and AMOVA (Analysis of Molecular Variance) analyses were conducted in Arlequin 3.5 to investigate genetic structure and gene flow, applying p-value corrections

using the B-Y method (Benjamini & Yekutieli, 2001; Narum et al., 2006). Using AMOVA to assess the distribution of genetic variability across hierarchical levels, populations were first analyzed in a grouped manner considering individual sampling locations. Subsequently, populations were grouped by watershed. For both approaches, we calculated proportions of genetic variation: among groups, among populations within groups, and within individuals, with significance values assessed through permutation tests.

Individuals were clustered into genetic populations using Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010) implemented in the *adegenet* package in R (version 4.2.3), and through the Structure software (version 2.3.3; Pritchard et al., 2000). The Structure analysis consisted of three runs of 500,000 generations each, with 50,000 generations of *burn-in* and K values ranging from 1 to 4. The most probable number of clusters was estimated using the methods of Evanno et al. (2005) and Puechmaille (2016) on the StructureSelector portal (Li & Liu, 2018), with results from the three runs combined using CLUMPAK (Kopelman et al., 2015). Contemporary gene flow was estimated in BayesAss3-SNPs (Wilson & Rannala, 2003; Mussmann et al., 2019) with five runs of 10 million MCMC iterations, sampled every 5,000 generations and with 2 million generations of *burn-in*. Run convergence assessment and result combination were subsequently performed in Tracer 1.7.2 (Rambaut et al., 2018).

## 2.4 RESULTS

### 2.4.1 Sequencing, assembly and filtering of SNPs

After sequencing the 92 *L. fortunei* samples from the Iguaçu River and Upper Paraná River basins, and applying filters for minimum allele frequency, missing SNPs and individuals, SNP spacing, Hardy-Weinberg equilibrium, and selective SNPs, two genetic databases were generated. Genetic database A (BG-A) prioritized including a greater number of SNPs and a smaller number of individuals, with 5,253 SNPs in 41 individuals: 4 from the Salto Caxias HPP reservoir, 10 from upstream of the Baixo Iguaçu HPP reservoir, 7 from the downstream population of Baixo Iguaçu HPP, and 20 from the Itaipu HPP reservoir. Genetic database B (BG-B) focused on a larger number of individuals but with fewer SNPs, totaling 2,422 SNPs in 77 individuals: 18 from the Salto Caxias HPP reservoir, 19 from upstream of the Baixo Iguaçu HPP reservoir, 17 from the downstream population of Baixo Iguaçu HPP, and 23 from the Itaipu HPP reservoir.

### 2.4.2 Genetic diversity of *L. fortunei* populations

The *L. fortunei* populations showed variable gene diversity among the analyzed locations (Tables 1 and 2). In the Salto Caxias HPP reservoir, gene diversity was 0.224 in BG-A and 0.167 in BG-B. For the populations upstream and downstream of Baixo Iguaçu HPP, the values were 0.195 (BG-A) and 0.163 (BG-B) for the upstream location, and 0.224 (BG-A) and 0.197 (BG-B) for the downstream location. In the Itaipu HPP reservoir, the indices were calculated as 0.202 in both BG-A and BG-B.

**Table 1.** Genetic diversity distribution of golden mussel populations from the Lower Iguaçu River basin and Itaipu HPP (Upper Paraná River). Number of genotyped individuals (N) and retained after filtering (NM) in genetic database A (BG-A), their respective proportions of missing data (NA), observed (HO) and expected (HE) heterozygosity, gene diversity (GD), number of private alleles (PA), inbreeding coefficient (FIS, all non-significant values), and effective population size (Ne, with 95% confidence interval). Ne α indicates the population is sufficiently large to show no evidence of linkage disequilibrium among analyzed SNPs.

| Population | *N* | $N_M$ | *NA* | $H_O$ | $H_E$ | *GD* | *PA* | $F_{IS}$ | *Ne* |
|---|---|---|---|---|---|---|---|---|---|
| Salto Caxias HPP reservoir | 25 | 4 | 20,5% | 0.149 | 0.182 | 0.224 | 56 | 0.138 | α (α - α) |
| Upstream Baixo Iguaçu HPP | 24 | 10 | 22,3% | 0.142 | 0.207 | 0.195 | 256 | 0.106 | α (α - α) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Downstream Baixo Iguaçu HPP | 19 | 7 | 19,3% | 0.146 | 0.199 | 0.224 | 148 | 0.127 | α (α - α) |
| Itaipu HPP reservoir | 24 | 20 | 17,8% | 0.145 | 0.228 | 0.202 | 2486 | 0.148 | α (2801.9 - α) |
| Global | 92 | 41 | 19,4% | 0.145 | 0.233 | 0.200 | | 0.133 | α (173.3 - α) |

**Table 2.** Genetic diversity distribution of golden mussel populations from the Lower Iguaçu River basin and Itaipu HPP (Upper Paraná River). Number of genotyped individuals (N) and retained after filtering (NM) in genetic database A (BG-B), their respective proportions of missing data (NA), observed (HO) and expected (HE) heterozygosity, gene diversity (GD), number of private alleles (PA), inbreeding coefficient (FIS, all non-significant values), and effective population size (Ne, with 95% confidence interval). Ne α indicates the population is sufficiently large to show no evidence of linkage disequilibrium among analyzed SNPs

| Population | *N* | $N_M$ | *NA* | $H_O$ | $H_E$ | *GD* | *PA* | $F_{IS}$ | *Ne* |
|---|---|---|---|---|---|---|---|---|---|
| Salto Caxias HPP reservoir | 25 | 18 | 16,1% | 0.158 | 0.200 | 0.167 | 37 | 0.072 | α (410.7 - α) |
| Upstream Baixo Iguaçu HPP | 24 | 19 | 17,4% | 0.157 | 0.198 | 0.163 | 27 | 0.079 | α (405.3 - α) |
| Downstream Baixo Iguaçu HPP | 19 | 17 | 16,6% | 0.156 | 0.203 | 0.197 | 26 | 0.093 | 417.6 (263.4 - 417.6) |
| Itaipu HPP reservoir | 24 | 23 | 8,1% | 0.166 | 0.207 | 0.202 | 405 | 0.108 | 408.2 (353.9 - 408.2) |
| Global | 92 | 77 | 14,2% | 0.160 | 0.210 | 0.166 | | 0.089 | 331.3 (260.2 - 353.1) |

At Salto Caxias HPP, observed heterozygosity (Ho) values were 0.149 (BG-A) and 0.158 (BG-B). For the upstream population of Baixo Iguaçu HPP, Ho was 0.142 (BG-A) and 0.157 (BG-B), while downstream it was 0.146 (BG-A) and 0.156 (BG-B). In the Itaipu HPP reservoir, Ho values were 0.145 (BG-A) and 0.166 (BG-B). Expected heterozygosity (He) showed a range of values within populations. At Salto Caxias HPP, He was 0.182 (BG-A) and 0.200 (BG-B). In the upstream and downstream populations of Baixo Iguaçu HPP, values were 0.207 (BG-A) and 0.198 (BG-B) for upstream, and 0.199 (BG-A) and 0.203 (BG-B) for downstream. At Itaipu HPP, values were higher compared to other locations, being 0.228 in BG-A and 0.207 in BG-B.

With respect to the inbreeding coefficients (FIS), the locations showed non-significant values. At Salto Caxias HPP, FIS values were 0.138 (BG-A) and 0.072 (BG-B), with no

significant evidence of inbreeding ($p > 0.05$). For the Baixo Iguaçu locations, values were 0.106 (BG-A) and 0.079 (BG-B) for the upstream site, and 0.127 (BG-A) and 0.093 (BG-B) for the downstream site. At Itaipu HPP, values were 0.148 (BG-A) and 0.108 (BG-B), with no evidence of inbreeding ($p > 0.05$). The Ne values estimated for BG-A and BG-B reflect demographic differences between the genetic databases (Tables 1 and 2)

2.4.3 Population structuring and connectivity among *L. fortune* populations

Molecular variance analyses (AMOVA) revealed low and non-significant "among populations" variation in *L. fortunei*, with 1.13% (BG-A, $p = 1.000$) and 0.72% (BG-B, $p = 0.999$), indicating no genetic structure among the analyzed populations. Most genetic diversity was attributed to the "within individuals" level, accounting for 77.32% (BG-A) and 87.93% (BG-B) of total variation. The "among individuals within populations" variation contributed 21.55% (BG-A) and 11.36% (BG-B) of the observed genetic diversity (Table 3). Regarding AMOVA results for *L. fortunei* populations grouped by watersheds, there was also no support for significant genetic structure among groups, with "among groups" variation representing only 2.81% (BG-A, $p = 0.239$) and 1.96% (BG-B, $p = 0.249$). Similarly, "among populations within groups" variation was negative (-0.49%, BG-A, $p = 0.728$ and -0.30%, BG-B, $p = 0.873$), reinforcing the absence of genetic structure among populations. Most genetic variation was observed "within individuals" (84.63%, BG-A, $p = 0.000$ and 89.51%, BG-B, $p = 0.000$), followed by "among individuals within populations" variation (13.05%, BG-A, $p = 0.000$ and 8.83%, BG-B, $p = 0.000$) (Table 3).

Global Fst for genetic database A (BG-A) was 0.011, and for genetic database B (BG-B) was 0.007, both not significantly different from zero, not supporting genetic differentiation among populations. Paired Fst values indicate varying levels of genetic differentiation among the studied populations (Table 4). The Fst values for both databases were non-significant among Lower Iguaçu River populations, while the Itaipu population showed Fst values significantly different from zero in comparisons with the former populations ($p < 0.05$).

**Table 3.** Results of Molecular Variance Analysis (AMOVA) for golden mussel populations from the Lower Iguaçu River basin and Itaipu HPP (Upper Paraná River) for genetic databases A (BG-A) and B (BG-B).

| Analysis | BG-A | | BG-B | |
|---|---|---|---|---|
| | Variation | p | Variation | p |
| Ungrouped populations | | | | |

| | | | | |
|---|---|---|---|---|
| Among populations | 1.13% | 1,000 | 0.72% | 0.999 |
| Among individuals within populations | 21.55% | 0.000 | 11.36% | 0.000 |
| Within individuals | 77.32% | 0.000 | 87,93% | 0.000 |
| Grouped populations in regions | | | | |
| Among groups | 2.81% | 0.239 | 1.96% | 0.249 |
| Among populations within groups | - 0.49% | 0.728 | -0.30% | 0.873 |
| Among individuals within populations | 13.05% | 0.000 | 8.83% | 0.000 |
| Within individuals | 84.63% | 0.000 | 89.51% | 0.000 |

**Table 4.** Paired FST values (below diagonal) and their respective p-values (above diagonal) for golden mussel populations from the Lower Iguaçu River basin and Itaipu HPP (Upper Paraná River), corresponding to genetic databases A (BG-A) and B (BG-B). (*indicates significant values after p-value correction using the B-Y method [$p < 0.024$])

| Population | BG-A | | | | BG-B | | | |
|---|---|---|---|---|---|---|---|---|
| | Salto Caxias HPP reservoir | Upstream Baixo Iguaçu HPP | Downstream Baixo Iguaçu HPP | Itaipu HPP reservoir | Salto Caxias HPP reservoir | Upstream Baixo Iguaçu HPP | Downstream Baixo Iguaçu HPP | Itaipu HPP reservoir |
| Salto Caxias HPP reservoir | | 0.234 | 0.810 | 0.009* | | 0.459 | 0.900 | 0.000 |
| Upstream Baixo Iguaçu HPP | 0.020 | | 0.864 | 0.000* | 0.002 | | 0.936 | 0.000 |
| Downstream Baixo Iguaçu HPP | 0.003 | 0.002 | | 0.000 | - 0.00088 | 0.002 | | 0.000* |
| Itaipu HPP reservoir | 0.022 | 0.036 | 0.027 | | 0.018 | 0.018 | 0.018 | |

The DAPC analyses indicate the formation of two main clusters among the analyzed populations (Figure 2). In both genetic databases (A and B), the populations from Baixo Iguaçu HPP (Upstream and Downstream) and Salto Caxias HPP formed a single cluster. In contrast, the Itaipu HPP population emerged as a distinct grouping, separate from the others. This pattern was consistent across both genetic databases (A and B).

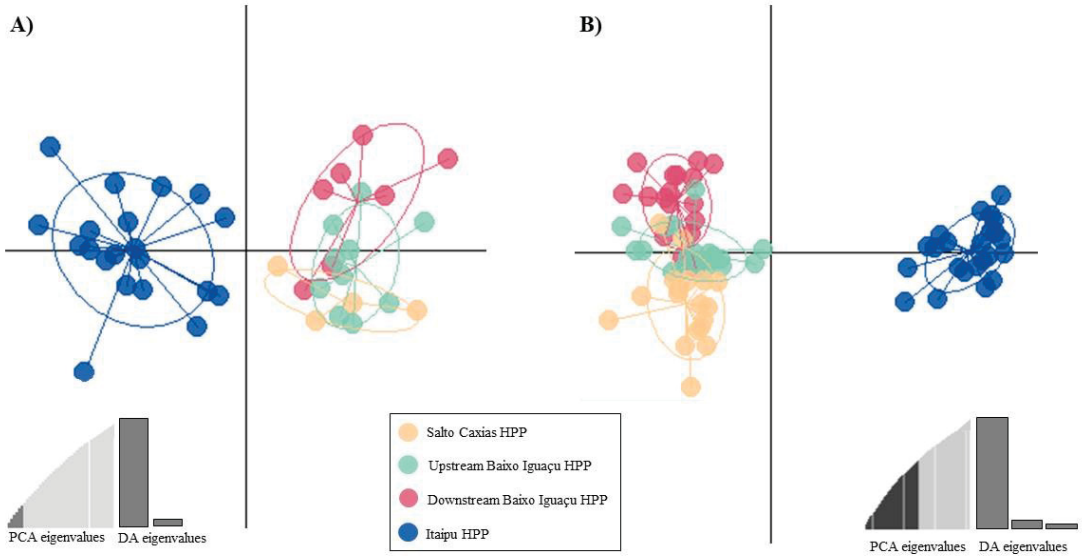


**Figure 2.** Genetic clusters of golden mussel populations from the Lower Iguaçu River basin (Salto Caxias HPP (yellow), Baixo Iguaçu HPP - Upstream (green), Baixo Iguaçu HPP - Downstream (red)) and Itaipu HPP (Upper Paraná River) (blue), obtained through DAPC (Discriminant Analysis of Principal Components) for genetic databases (A) BG-A and (B) BG-B.

The Structure clustering analyses support groupings similar to those obtained by DAPC. For genetic database A, the most probable number of clusters was 2 according to the Evanno et al. (2015) test and 3 per the Puechmaille (2016) test, while for genetic database B, the most probable number of clusters was 2 in both tests (Figure 3). In the analysis with two clusters (K = 2), individuals from the Lower Iguaçu River locations were grouped into a single genetic cluster, while those from the Itaipu HPP reservoir formed a distinct grouping in both databases.

In the analysis with three clusters (K = 3), the results were congruent for both genetic databases (A and B), showing the formation of two distinct clusters among the Lower Iguaçu River populations compared to the Itaipu HPP population, which remained isolated.

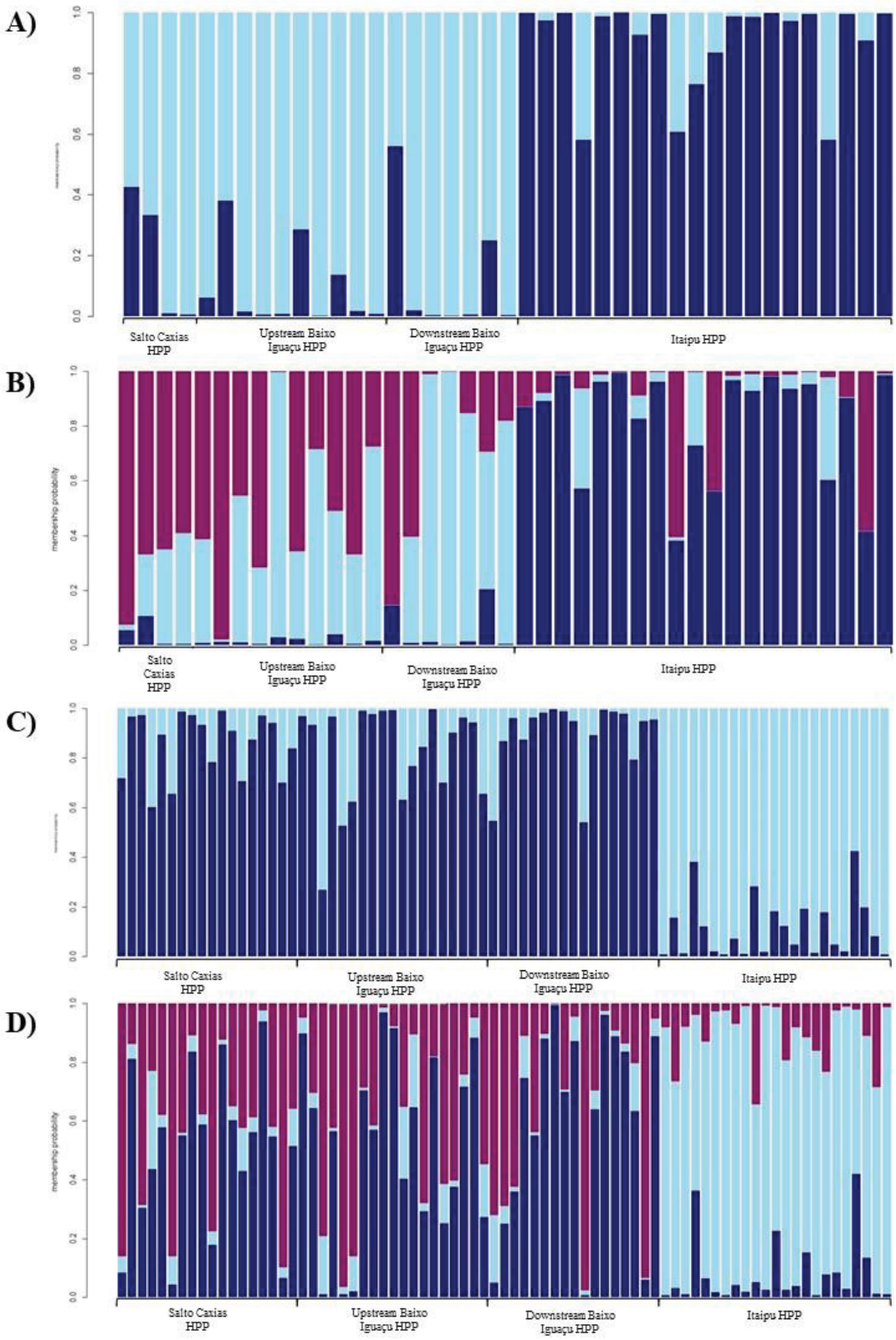


**Figure 3.** Genetic clusters of golden mussel individuals from populations of the Lower Iguaçu River basin and Itaipu HPP (Upper Paraná River), in Structure analyses corresponding to genetic database A (A and B) and B (C and D), clustered into two (K = 2) and three (K = 3) groups. Each column represents an individual, and each color indicates the proportion of its genome derived from each genetic cluster.

The contemporary gene flow analyses (Table 5) reveal a scenario marked by pronounced genetic isolation of the Itaipu reservoir, which shows high proportions of non-migrants (95.8% in BG-A and 96.3% in BG-B), contrasting with the connected dynamics observed along the Iguaçu River. In this system, the flow patterns show interesting asymmetries: while BG-A shows reciprocal exchanges between Salto Caxias and the other populations (contributing 2.3% to Upstream Baixo Iguaçu HPP and 3.0% to Downstream Baixo Iguaçu HPP, while receiving 4.1% from both Upstream and Downstream), BG-B highlights a more intense flow from Upstream to Downstream Baixo Iguaçu HPP (4.6%) compared to the reverse movement (1.7%). The flows observed between adjacent populations (such as the 3.5% from Upstream Baixo Iguaçu HPP to Salto Caxias in BG-B) may reflect both geographic proximity and local demographic processes, considering the absence of data from more upstream populations (e.g., Osório region). The predominance of upstream-downstream flow in BG-B (4.6% vs 1.7%) follows the expected pattern for passive larval dispersal, while the reciprocal exchanges in BG-A suggest a more complex dynamic, possibly influenced by the local hydrological system configuration.

**Table 5.** Estimated percentage of migrants and non-migrants in golden mussel populations from the Lower Iguaçu River Basin and Itaipu HPP (Upper Paraná River), considering genetic databases A (BG-A) and B (BG-B). Rows represent recipient populations, while columns indicate donor populations.

| Population | BG-A | | | | BG-B | | | |
|---|---|---|---|---|---|---|---|---|
| | Salto Caxias HPP reservoir | Upstream Baixo Iguaçu HPP | Downstream Baixo Iguaçu HPP | Itaipu HPP reservoir | Salto Caxias HPP reservoir | Upstream Baixo Iguaçu HPP | Downstream Baixo Iguaçu HPP | Itaipu HPP reservoir |
| Salto Caxias HPP reservoir | 87,5% | 4,1% | 4,1% | 4,1% | 93,5% | 3,5% | 1,5% | 1,5% |
| Upstream Baixo Iguaçu HPP | 2,3% | 92,9% | 2,3% | 2,3% | 2,9% | 92,9% | 2,2% | 1,9% |
| Downstream Baixo Iguaçu HPP | 3,0% | 3,0% | 90,9% | 3,0% | 1,7% | 4,6% | 91,9% | 1,7% |
| Itaipu HPP reservoir | 1,3% | 1,3% | 1,3% | 95,8% | 1,2% | 1,2% | 1,2% | 96,3% |

## 2.5 DISCUSSION

The results reveal the current genetic scenario of *Limnoperna fortunei* populations in the Iguaçu basin and Itaipu. The gene diversity (GD), ranging between 0.163 and 0.224, associated with heterozygote deficiency - but without evidence of significant inbreeding ($p > 0.05$) - suggests that recent demographic processes may have influenced the current genetic structure. Comparison among studied populations shows variation in gene diversity levels, a pattern consistent with expectations for populations established from few introduced individuals, as observed in other systems (Belz et al., 2012; Borges et al., 2017). Additionally, the absence of inbreeding evidence in downstream populations of Baixo Iguaçu HPP and Salto Caxias HPP reservoir ($p > 0.05$) suggests that, despite heterozygote deficiency, no significant inbreeding was detected in the analyzed populations.

The genetic data reveal gene diversity values (GD = 0.163-0.224) and reduced heterozygosity in the analyzed populations. This pattern may reflect demographic processes characteristic of invasive species during their establishment phase. The relative genetic homogeneity observed along the Iguaçu River is consistent with anthropogenic dispersal between reservoirs, as demonstrated by Belz et al. (2012) for the transport of individuals via sand used in beach construction projects along river sections. Furthermore, passive larval dispersal along the natural hydrological gradient (upstream-downstream) between the Iguaçu HPPs appears to contribute to the observed connectivity.

The genetic distinction between Itaipu and Iguaçu populations, evidenced by DAPC and Structure analyses, reinforces the role of Iguaçu Falls as an effective geographic barrier, preventing or minimizing genetic exchange between populations in the downstream-upstream direction. However, the low genetic structure observed within the Iguaçu system itself suggests that once the initial barrier is overcome, secondary dispersal occurs through different models: (i) Jump dispersal facilitated by anthropogenic activities (e.g., sand transport) (Belz et al., 2012; Zhan et al., 2012), and (ii) Natural dispersal along the river, particularly for larval and juvenile stages, which are carried by currents at local scales following the hydrological flow. This combination of mechanisms would explain the genetic connectivity between upstream and downstream subpopulations of Baixo Iguaçu HPP and Salto Caxias HPP, even in a system with partial barriers.

### 2.5.1 The current scenario

Current findings demonstrate that the observed genetic patterns align with previous studies on *L. fortunei* invasion genetics. Zhan et al. (2012), analyzing introduced populations

in South America, reported lower-than-expected heterozygosity (Fis between 0.079 and 0.148) without evidence of inbreeding - a pattern similar to that detected in this study. Ghabooli et al. (2013) and Uliano-Silva et al. (2017) emphasize that habitat fragmentation and successive founder events may influence genetic diversity. In the Iguaçu River system, the absence of significant genetic structure between upstream and downstream HPP populations suggests these dams do not act as effective dispersal barriers for golden mussels, unlike observations for native fish (Agostinho et al., 2007). This conclusion is reinforced by the species' high adaptability to reservoirs, where it frequently exhibits population explosions (Boltovskoy et al., 2006; Darrigran & Damborenea, 2009).

Assessment of different Brazilian watersheds by Ludwig et al. (2021) demonstrated that *L. fortunei* populations maintain stable levels of genetic diversity, even across different hydrological systems. Our results support this observation, indicating that populations in the Iguaçu River exhibit genetic diversity comparable to other systems invaded by the species. The low genetic structure among subpopulations suggests efficient gene flow along the river, mediated both by passive larval dispersal in the upstream-downstream direction and by anthropogenic transport (Dlugosch & Parker, 2007; Ghabooli et al., 2010). This pattern contrasts with what is expected in natural migrations of native species, which typically show more defined spatial genetic structure (Allendorf & Lundquist, 2003; Zhan et al., 2012). The genetic distinction between Itaipu and Iguaçu reinforces the role of Iguaçu Falls as a geographic barrier, while the connectivity observed within the Iguaçu system indicates that dispersal mechanisms have contributed to the genetic dynamics of established populations (Ghabooli et al., 2013).

The DAPC and Structure analyses reveal a clear genetic distinction between Itaipu and Iguaçu populations, a pattern consistent with previous studies (Zhan et al., 2012; Borges et al., 2017; De Paula et al., 2020; Ludwig et al., 2021). Considering that: (1) the Itaipu reservoir was one of the first invaded locations in the region (Belz et al., 2012); and (2) the geographical proximity between systems, it is plausible that Iguaçu populations became established from propagules originating in Itaipu or the Middle Paraná, although our data do not permit direct testing of this hypothesis (Belz et al., 2012; Borges et al., 2017). The relatively lower genetic variability observed in Iguaçu compared to Itaipu may reflect both the founder effect during initial colonization, followed by natural downstream dispersal complemented by anthropogenic vectors among subpopulations along the Iguaçu (Belz et al., 2012; Ludwig et al., 2021). This scenario aligns with invasive species studies demonstrating how natural barriers can create genetic structure between isolated systems, while

anthropogenic dispersal mechanisms maintain connectivity in interconnected systems (Saastamoinen et al., 2017).

Contemporary gene flow patterns reveal a distinct dynamic between populations. The high proportion of non-migrants in Itaipu (96.3% in BG-B) indicates genetic isolation in this population, whereas in Baixo Iguaçu, intense genetic exchange among subpopulations is observed. The data reveal bidirectional movements between Upstream and Downstream regions of Baixo Iguaçu, with a greater contribution from Upstream to Downstream (4.6% in BG-B). This asymmetry may reflect the combined influence of river hydrodynamics and the absence of significant genetic differentiation between these subpopulations. The observed structuring between Itaipu and Baixo Iguaçu corroborates previous studies on the species' genetic diversity in the region (Borges et al., 2017; Ludwig et al., 2021).

The integration of contemporary gene flow results with DAPC/Structure analyses reveals a scenario consistent with an invasion process mediated by multiple dispersal mechanisms. The genetic similarity among Iguaçu populations (DAPC/Structure), coupled with intense gene flow between them, suggests that current connectivity along the river is maintained by both natural and anthropogenic mechanisms (Belz et al., 2012; Zhan et al., 2012; Borges et al., 2017). The genetic distinction of Itaipu, evident in all analyses, indicates relative isolation of this system. This pattern—with Iguaçu populations being genetically homogeneous yet distinct from Itaipu—reflects the characteristic dynamics of invasive species in fragmented systems, as described in studies on the genetic structure of invasive mollusks (Ghabooli et al., 2013; Ludwig et al., 2021).

A low number of initial introduction events could, in theory, generate differentiation among Iguaçu populations and, over time, undergo a homogenization process through natural dispersal along the fluvial gradient (Borges et al., 2017). The current genetic connectivity among populations primarily reflects larval hydrological dispersal, potentially complemented by anthropogenic activities. This scenario is consistent with gene flow estimates, particularly with the higher number of migrants detected in the downstream population, following the expected pattern for predominantly downstream dispersal in riverine systems. The current dynamics of Iguaçu populations thus appear to be shaped mainly by processes of continuous natural dispersal, while Itaipu maintains distinct genetic characteristics.

### 2.5.2 The advance of *L. fortunei* since Borges et al. (2017) in the Iguaçu River

The study by Borges et al. (2017), conducted at eight sampling points along the Iguaçu River (covering its lower, middle, and upper sections) and in the Itaipu Hydroelectric Power

Plant reservoir (Iguaçu River mouth/Paraná River) between 2012 and 2013, investigated the population dynamics of *L. fortunei* in the region using mitochondrial DNA (mtDNA COI) sequences to assess genetic variability in both larvae and adults, while also testing the hypothesis about the origin of the Iguaçu River populations. The authors suggested that the species introduction occurred through anthropogenic events, with dispersal mediated by hydrological flow and the absence of significant barriers to larval movement. Our results, based on SNP markers via NGS, not only corroborate but also expand these conclusions.

The genetic differences between Itaipu and Iguaçu populations, attributed either to the natural barrier of Iguaçu Falls or to the species' introduction mode, remain evident even after nearly a decade (Belz et al., 2012; Borges et al., 2017; Ludwig et al., 2021). The current patterns of genetic connectivity in the Iguaçu system are consistent with the observations of Borges et al. (2017), who had already identified gene flow among populations in the section where the Baixo Iguaçu Hydroelectric Power Plant (HPP) would later be constructed. Our data reinforce this interpretation, showing that the analyzed populations maintain an absence of genetic structure, suggesting ongoing individual movement between regions, likely mediated by both natural dispersal processes along the fluvial gradient and human activities.

In the Lower Iguaçu River, Borges et al. (2017) observed that adult populations did not exhibit distinct genetic structure - a pattern consistent with maintained connectivity among subpopulations. Our results corroborate the absence of genetic structure in this region, indicating that the previously described homogenization process persists. The genetic diversity observed in our samples is compatible with expectations for established *L. fortunei* populations in similar systems (Ludwig et al., 2021), although direct temporal comparison is limited by methodological differences between studies. The overall pattern suggests that population dynamics in the region continue to be influenced primarily by the species' characteristic natural dispersal mechanisms, complemented by gene flow among connected subpopulations.

An interesting contrast emerges when comparing population structure patterns. While Borges et al. (2017) detected some structure along the Iguaçu River, our data reveal a more homogeneous scenario without evident structuring. This divergence may reflect both the higher resolution of SNP markers and actual changes in population dynamics. The high genetic connectivity observed among Lower Iguaçu populations, coupled with the absence of effective barriers to larval dispersal, suggests increased genetic exchange mediated by both passive downstream dispersal and accidental transport via anthropogenic activities (Zhan et al., 2012; Ghabooli et al., 2013). This genetic homogeneity pattern aligns with those described

for golden mussel populations in other hydrological systems (Ludwig et al., 2021), where the combination of natural and human factors maintains the observed population structure. The genetic differentiation between Itaipu and Iguaçu persists even after nearly a decade since initial invasion records, suggesting that significant new introductions - which could have reduced this differentiation - likely did not occur during this period.

## 2.6 FINAL CONSIDERATIONS

This study revealed that *Limnoperna fortunei* populations in the Iguaçu basin and Itaipu Reservoir exhibit genetic dynamics influenced by both historical processes (including initial colonization from limited sources) and contemporary dispersal patterns (encompassing both active and passive dispersal), with implications for understanding their invasion and persistence in the region. The genetic distinction between Itaipu and Iguaçu reinforces the role of Iguaçu Falls as an effective geographic barrier, while the observed homogeneity along the Iguaçu's main course indicates connectivity maintained by both anthropogenic vectors (boat traffic, sport fishing) and natural mechanisms (passive downstream larval dispersal).

The results reveal distinct patterns between basins: while the Iguaçu system maintains high connectivity among populations, Itaipu retains a differentiated genetic signature despite its potential role as an introduction source. This dynamic suggests that management strategies should be context-specific, incorporating both physical barriers and control of anthropogenic dispersal vectors (e.g., inter-reservoir vessel traffic). Furthermore, ongoing genetic monitoring could help detect new introductions and dispersal routes, aiding containment of *L. fortunei* expansion into adjacent basins.

In summary, this work underscores the importance of integrating genetic approaches to understand invasive species dynamics in these ecosystems. The patterns observed in *L. fortunei* reinforce those biological invasions are dynamic processes shaped by both natural factors and human activity, requiring adaptive management strategies that incorporate evolving knowledge about their biology and the dispersal mechanisms that sustain them.

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## CONCLUSÕES GERAIS

O presente estudo fornece uma análise abrangente da estrutura genética e dos processos evolutivos que moldam as populações invasoras de *Limnoperna fortunei* na bacia do Rio Iguaçu e na UHE Itaipu, integrando dados genômicos de alta resolução com inferências ecológicas e históricas. Os resultados obtidos revelam um cenário complexo, no qual padrões de diversidade genética, fluxo gênico e estruturação populacional refletem a influência da atividade humana (através de vetores como navegação e atividades de piscicultura) quanto processos naturais de dispersão ao longo do gradiente fluvial, com evidências de padrões hierárquicos de colonização rio abaixo.

Um dos achados centrais deste trabalho é o reforço da hipótese do papel das Cataratas do Iguaçu como uma barreira geográfica efetiva, responsável pela distinção genética entre as populações de Itaipu e Iguaçu. No entanto, o fluxo gênico entre estas populações sugere que mecanismos antrópicos — como o transporte involuntário de larvas ou adultos em embarcações — podem estar promovendo um fluxo gênico secundário que conecta subpopulações distintas (Belz et al., 2012, Borges et al., 2017, Ludwig et al., 2021).

Apesar dos sinais de eventos passados de gargalo populacional, as populações de *L. fortunei* no Iguaçu demonstram uma notável capacidade de persistência. Essa resiliência parece ser sustentada por dois fatores principais: (1) a plasticidade fenotípica da espécie, que lhe permite prosperar nas condições atuais, e (2) a conectividade genética entre subpopulações ao longo do rio, que compensa os efeitos negativos da deriva genética e mantém a variabilidade necessária para a adaptação local (Zhan et al., 2012, Borges et al., 2017).

Nossos resultados revelam que, dentro da bacia do Iguaçu, as populações de *L. fortunei* apresentam conectividade e fluxo gênico que sustentam sua variabilidade genética (Zhan et al., 2012; Ferreira et al., 2024). A diferenciação entre Itaipu e o Iguaçu persiste, indicando que as Cataratas ainda atuam como barreira geográfica relevante. Essa configuração pode refletir tanto a origem comum das populações (com Itaipu como possível fonte histórica para o Iguaçu) quanto os atuais padrões de isolamento. Essa dinâmica tem implicações diretas para estratégias de manejo. Medidas convencionais de controle, como barreiras físicas ou tratamentos químicos localizados, podem se tornar progressivamente menos eficazes à medida que a conectividade entre subpopulações aumenta (With, 2002; Boltovskoy et al., 2015; Darrigran et al., 2020). Essa limitação é particularmente relevante em bacias com múltiplos reservatórios, onde a dispersão passiva de larvas e adultos é facilitada pela dinâmica hidrológica (With, 2002).

Do ponto de vista aplicado, nossos resultados destacam a necessidade de políticas de gestão que considerem os aspectos ecológicos e antrópicos do processo de invasão. A implementação de protocolos de descontaminação de embarcações, a fiscalização do transporte de materiais entre reservatórios e o monitoramento genético contínuo das populações devem ser priorizados para retardar a expansão da espécie. Além disso, a identificação de regiões com alta diversidade genética pode ajudar a direcionar esforços de controle para fontes prioritárias de propágulos.

Em uma perspectiva mais ampla, este estudo ilustra como invasões biológicas em ecossistemas fragmentados são moldadas pela interação entre fatores naturais e antrópicos. A capacidade de *L. fortunei* de aproveitar rotas de dispersão criadas pelo homem — seja através de hidrovias, barragens ou transporte comercial — transforma-o em um modelo emblemático dos desafios impostos pela globalização aos ecossistemas aquáticos continentais (Darrigran & Damborenea, 2009; Gallardo et al., 2015). Se, por um lado, a genômica populacional nos permite reconstruir a história dessas invasões, por outro, ela também aponta caminhos para intervenções mais informadas e eficazes.

Por fim, este trabalho reforça que a contenção de espécies invasoras como *L. fortunei* exige uma abordagem multidisciplinar, combinando pesquisa básica, monitoramento ambiental e cooperação institucional. À medida que as pressões antrópicas sobre os ecossistemas aquáticos se intensificam, estudos que integrem padrões genéticos, processos ecológicos e dimensões socioambientais serão cada vez mais cruciais para equilibrar desenvolvimento e conservação nos ecossistemas fluviais.

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